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# A high-throughput screening strategy for accurate quantification of erythromycin

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ABSTRACT

A simple and rapid microplate-based bioassay was developed for the quantitative assay of erythromycin in *Saccharopolyspora erythraea* fermentation samples. High performance liquid chromatography (HPLC), colorimetric method and agar diffusion assay (ADA) were used as reference methods to evaluate the accuracy of this strategy. *S. erythraea* T-13 and its derivatives were used to assess the optimum assay conditions for erythromycin measurement and it was verified that this downscaled method was comparable to traditional assay methods for rapid quantification. Large number of isolates could be evaluated simultaneously within 4–5 h by using the high-throughput method. The cost effective method reported here demonstrates the successful application of a microplate assay for high throughput analysis, and could be potentially applicable to general antibiotics analysis.



## 1. Introduction

Erythromycin is a macrolide antibiotic produced by actinomycete *Saccharopolyspora erythraea* (*S. erythraea*) that is used primarily against gram-positive bacteria, although first used in 1952, it is still one of the most commonly-used antibiotics and it has proved to be a safe and effective treatment for a number of common infections, not only in humans, but also in food-producing animals to control bacterial diseases and promote animal growth [1]. There existed four main components (erythromycin A, B, C and D) in erythromycin products. Erythromycin A (Er-A) is the most potent and clinically important component. Erythromycin B (Er-B), erythromycin C (Er-C) and Erythromycin D (Er-D) were existed as the intermediates in the biosynthesis pathway of Er-A [2,3], which were usually regarded as the by-products in fermentation process.

The success of any strain improvement programme mainly depends on the number of positive isolates that can be screened after mutagenic treatment [4,5]. As the demand is increasing, it is important and preferential to establish a simple and accurate high-throughput analytical method for screening libraries of isolates to obtain high-production strains. There are many techniques for the quantification of erythromycin [6], such as thin-layer (TLC) and paper chromato-graphic (PC), gas–liquid chromatography, high-performance liquid chromatography (HPLC). Among these, HPLC is the most extensively used chromatographic method for the analysis of erythromycin and related substances. However, it is so tedious and expensive that is impractical for high-throughput assay. There is a considerable interest to employ bioassay to estimate the bioactivity. However, one major difficulty is obtaining accurate and precise quantification using bioassays [7–9]. The agar diffusion assay published by [10] and modified later by [11] is still the preferred method for most antibiotics quantifications [12]. But, it is time consuming and laborious, requiring preparation and cooling of plates, boring of test wells in agar and manual measuring of inhibition zones after 24–48 h incubation [10]. Results depend mainly on human ability and judgment and the suggested precision cannot be obtained when the inhibition zone is unclear or not perfectly circular [13]. Over the last 2 decades, new alternatives for testing antimicrobial activity have been proposed, including ATP-bioluminometry, radiometry, conductance measurement and automated turbidometry [14–17]. These techniques have not gained wide acceptance possibly because they require dedicated equipments and supplies operator. Therefore, a rapid assay method that can evaluate large number of isolates simultaneously with a fair degree of accuracy and reproducibility becomes imperative [18–20].

Turbidimetry is a simple way to correlate bacterial growth with inhibitory effect by optical density measurement. The limitations of traditional tube turbidimetry was mainly due to the effect of time between inoculation and reading of the tubes, low sensitivity and the large material requirement [21,22]. Erythromycin could effectively inhibit the growth of *Bacillus pumilus*. In this study, a rapid and simple high-throughput microplate bioassay based on turbidimetry was developed for erythromycin quantification in the fermentation broth. The assay was tested for its accuracy and precision using three reference methods (HPLC, ADA and colorimetric method). Furthermore, the optimum detection range

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of sensitive antibiotics concentration, added volume of indicator bacteria suspension, and the incubation time were investigated for high-throughput bioassay. Hundreds of samples can be analyzed simultaneously, and the data can be obtained well within 4–5 h, so the improved microplate bioassay could take the place of these tedious and time-consuming conventional protocols completely, particularly the well-diffusion method. To the best of our knowledge, this is the first report on high-throughput bioassay for quantification of total erythromycin production.

## 2. Materials and methods

### 2.1. Microorganisms

*S. erythraea* (T-13), has been deposited at China General Microbiological Culture Collection Center with the accession number of CGMCC 4.197, and the mutants derived from T-13 were grown on agar medium for 7 days (per liter: 10.0 g starch, 12.0 g corn steep liquor, 3.0 g NaCl, 3.0 g $(NH4)_2SO_4$, 2.0 g $CaCO_3$, 18.0 g agar; pH 6.5); *B. pumilus* CGMCC 1.1625 has been deposited at China General Microbiological Culture Collection Center with the accession number of CGMCC 1.1625, the indicator organism of bioactivity assay for erythromycin, grown on agar medium (per liter: 5.0 g peptone, 3.0 g beef extract, 3.0 g $K_2HPO_4$, 15.0 g agar; pH 8.2). All the microorganisms are preserved in our laboratory at 4 °C.

### 2.2. Media

Seed medium (per liter): 30.0 g starch, 18.0 g soybean flour, 3.0 g NaCl, 13.0 g corn steep liquor, 1.2 g $(NH4)_2SO_4$, 1.2 g $NH_4NO_3$, 6.0 g $CaCO_3$, 2.0 ml soy oil, pH 7.0; fermentation medium (per liter): 20.0 g starch, 30.0 g soybean flour, 30.0 g dextrin, 2.0 g $(NH4)_2SO_4$, 6.0 g $CaCO_3$, 10.0 ml soy oil, pH 7.2; Bioactivity assay medium for erythromycin (per liter): 5.0 g peptone, 3.0 g beef extract, 3.0 g $K_2HPO_4$, pH 8.2.

### 2.3. Growth conditions

*S. erythraea* (T-13) and its mutants were precultured in 500-ml shake flask containing 50 ml seed medium at 32 °C on a rotary shaker at 220 rpm (50 mm shaking diameter, 30/300, ZHWY-3212, China) for 48 h. 10% (v/v) precultures were inoculated in 500-ml shake flask containing 50 ml fermentation medium at 32 °C, 220 rpm. The samples were taken every 24 h and centrifuged at 3000 × *g* for 30 min. The supernatant was used to analyze the bioactivity.

*S. erythraea* (T-13) and its mutants were inoculated with toothpicks in 48-deep microtiter plates (48-deep MTPs) containing 500 μl seed medium at 32 °C on a rotary shaker at 220 rpm for 48 h. 10% (v/v) precultures were inoculated in the corresponding wells of new 48-deep MTPs containing 1.0 ml fermentation medium at 32 °C and 220 rpm until the end of the fermentation. The samples were harvested every 24 h and the supernatant was collected by MTP centrifuge (TDZ5-WS, China; 3000 × *g*, 30 min). The supernatant was used to analyze the bioactivity.

### 2.4. High-throughput bioactivity assay for antibiotics

#### 2.4.1. Preparation of indicator bacteria suspension

*B. pumilus* CGMCC 1.1625 was grown on fresh slants for 24 h. Cells were harvested with 0.9% sterile saline, the concentration of cell suspension was measured by microplate reader (Multiskan MK3, Thermo). *B. pumilus* CGMCC 1.1625 had a maximum ultraviolet absorbance at 580 nm. The concentration of the fresh cell suspension was diluted to an optical density (OD) of 1.0, and 5% (v/v) indicator bacteria suspension was added into the bioactivity assay medium.

#### 2.4.2. Sample assay

25 μl diluted samples were added into the 96-well MTPs contained 225 μl bioactivity assay medium mixed with indicator bacteria suspension beforehand, then cultured at 37 °C with a shaking at 180 rpm (TAITEC MBR-420FL, Japan) until OD value of the control sample reached 0.7 (4–5 h). Then, the 96-well MTPs were measured by microplate spectrophotometer immediately. The wells without antibiotics were treated as control sample. The wells with bioassay medium were treated as blank control. In order to eliminate the turbidity effect of medium on the measurement, the actual OD value of samples were the measured OD value subtracted the OD value of blank control. Three replicate wells were employed to test each sample concentration. The detailed procedure was illustrated in Fig. 1.

### 2.5. Other analytical methods

#### 2.5.1. HPLC

HPLC (Agilent 1100 Series, China) was used as reference method I for detection of erythromycin. The samples were separated on a Hypersil BDS-C18 column (4 mm × 250 mm, 5 μm, Elite, China), and eluted by $K_2HPO_4$ (0.02 M)/acetonitrile (45:55, v/v) at a flow rate of 1 ml/min, 60 °C, and 20 μl of sample was taken with automatic injector and measured at 215 nm by a UV detector.

#### 2.5.2. Colorimetric method

Colorimetric method was used as reference method II for detection of erythromycin. 2 ml diluted sample and 8 ml 0.35% $K_2CO_3$ were mixed in a separating funnel, 10 ml butyl acetate were added and shaked for 30 s, discarded the bottom layer liquid, 1.0 g anhydrous sodium sulfate were added and shaken until the solution was transparent, 5 ml supernatant liquid was transferred to a test tube with stopper, shaked with 10 ml HCl (0.1 M) for 30 s, rested until stratification, 5 ml bottom layer liquid was transferred to a dry test tube, added 5 ml $H_2SO_4$ (8 M) and kept warm together at 50 °C for 30 min, detected at 483 nm with a spectrophotometer.

#### 2.5.3. Agar diffusion assay (ADA)

ADA method was used as reference method III for detection of erythromycin. Medium for ADA (per liter): 5.0 g peptone, 3.0 g beef extract, 3.0 g $K_2HPO_4$, 15.0 g agar, pH 8.2. Assays were routinely performed in 90 mm Petri dishes containing 20 ml of nutrient agar in the bottom layer. Surface layer was kept liquid at 40–45 °C, an overnight culture of the indicator strain suspension ($OD_{580}$ = 1.5) was then added at a final concentration of 2% (v/v) and precisely 5 ml of this inoculated sloppy agar was applied uniformly over the bottom layer and the plates were allowed to set for 30 min at room temperature. Six wells were bored in each plate using the wide end of a sterile Pasteur pipette. 100 μl erythromycin standard solution (1, 2, 3, 4, 5, 6, 7, 8, 9 μg/ml) was dispensed to each well, each concentration was replicated three times. The plates were then incubated at 37 °C for at least 24 h to give a well-defined inhibition zone. Inhibition zone diameters were measured to the nearest 0.1 mm using a calliper. The diameter of the inhibition zone around each well was measured horizontally and vertically, and the mean of the largest and shortest diameter was calculated. Diameters of inhibition zones vs $\log_{10}$ erythromycin concentrations were plotted in order to obtain a standard curve. Samples were treated similarly and concentrations of antibiotic determined by interpolation on the standard curve.